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<b>(21) International Application Number:</b> PCT/US96/07375 <b>(22) International Filing Date:</b> 21 May 1996 (21.05.96)  <b>(30) Priority Data:</b> 08/447,277                      22 May 1995 (22.05.95)                      US  <b>(71) Applicant:</b> THE REGENTS OF THE UNIVERSITY OF CALIFORNIA [US/US]; 22nd floor, 300 Lakeside Drive, Oakland, CA 94612 (US).  <b>(72) Inventor:</b> SADEE, Wolfgang; 125 Lagunitas, Ross, CA 94957 (US).  <b>(74) Agents:</b> SIEBERT, J., Suzanne et al.; Majestic, Parsons, Siebert & Hsue, Four Embarcadero Center, Suite 1450, San Francisco, CA 94111-4121 (US).		<b>(81) Designated States:</b> CA, JP, Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).  <b>Published</b> <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
<b>(54) Title:</b> METHODS FOR G PROTEIN COUPLED RECEPTOR ACTIVITY SCREENING		
<b>(57) Abstract</b> <p>A method for screening G protein coupled receptors is provided in which G protein coupled receptors that are constitutively active are determined, such as by measuring receptor phosphorylation agonist independent signalling. When a G protein coupled receptor is found to be regulated by constitutive activity, then assay systems may be set up to classify test compounds as agonists, neutral antagonists, or negative antagonists with respect to G protein coupled receptor signalling and phosphorylation. Such determinations and screening are useful for selecting new pharmaceuticals potentially useful in treating disease states mediated by G protein coupled receptors, with applications including treatments in conjunction with narcotic analgesia.</p>		

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**METHODS FOR G PROTEIN**  
**COUPLED RECEPTOR ACTIVITY SCREENING**

**Field of the Invention:**

5           The present invention generally relates to G  
protein coupled receptors, and more particularly to  
assays for determining certain activities of G protein  
receptors, which determinations may be applied to  
modulating the activities of G protein coupled receptors  
10 for therapeutic benefit.

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**Background of the Invention**

20           G protein coupled receptors (hereinafter  
sometimes termed "GPCR"s) comprise a large superfamily  
of receptors sharing a common structural motif of seven  
transmembrane helical domains. When a ligand (an  
agonist) binds to a GPCR and activates it, signal  
25 transduction is achieved through the intermediary G  
protein (a heterotrimeric GTP binding protein) which in  
turn activates the second messenger system. Although  
the exact nature of the receptor-G protein interactions  
is not yet known, the receptor activated regulatory

cycle of the G protein involves GTP exchange for GDP, dissociation of the  $\alpha$  and  $\beta\gamma$  subunits, activation of the second messenger pathway by GTP- $G_\alpha$  and  $\beta\gamma$ , and termination of activation upon GTP hydrolysis to GDP by the inherent GTPase activity of the  $\alpha$  subunit. G protein coupled receptors regulate virtually all bodily functions ranging from vision and olfaction to neuronal and endocrine signaling.

A general property of signal transduction mediated by G protein coupled receptors is the attenuation of signaling upon prolonged agonist stimulation. These processes are referred to as desensitization, tachyphylaxis, adaptation, tolerance, or quenching. Because signal attenuation limits the clinical uses of many pharmaceuticals acting on GPCRs, the mechanism for this process has been the focus of much research. Receptor phosphorylation by selective kinases of G protein coupled receptors (termed "GRK"s) has been shown to contribute to desensitization of several receptors. To date, no selective and or potent GRK inhibitors have been reported, other than heparin which does not penetrate into intact cells, even though such inhibitors might prevent desensitization in these cases (e.g., the  $\beta_2$  receptor). GRKs selectively polyphosphorylate only the active receptor state, which not only serves as a preferred substrate, but also directly stimulates GRK activity.

Another emerging recognized feature of a number of GPCRs is the presence of a basal level of signalling activity, occurring in the absence of any agonist ligand. Mutations inducing high basal activity have been associated with genetic disorders, demonstrating the physiological relevance of basal receptor activity. For GPCRs displaying basal activity, two classes of antagonist have been defined, i.e.,

neutral antagonists which block only agonist induced effects without changing basal activity, and inverse agonists, or negative antagonists, which also block basal receptor activity.

5           Prior assay attempts to detect any significant changes of the  $\mu$  opioid receptor system during prolonged agonist exposure have been unable to determine biochemical mechanisms underlying narcotic addiction. Thus, much of the current research work has focused on  
10 events downstream of the receptor, such as long-term gene regulation, in attempting to account for the dependent state. Because the dependence liability of narcotic drugs severely limits their clinical utility as potent analgesics and exerts a heavy toll on society  
15 through illicit narcotic drug use, a screen for agents that could prevent or reverse the narcotic dependent state or might facilitate gradual withdrawal would greatly enhance the clinical utility of narcotic analgesics and could serve as an effective  
20 pharmacological weapon in the fight against illicit drug use.

#### Summary of the Invention

The present invention is directed to certain G protein coupled receptors where exposure to agonist  
25 leads to persistent constitutive activation of the receptors by phosphorylation.

G protein coupled receptors that are within the scope of this invention include all subtypes of the opioid, muscarinic, dopamine, adrenergic, cAMP, opsins,  
30 angiotensin, serotonin, thyrotropin, gonadotropin, substance-K, substance-P and substance-R receptors, melanocortin, metabotropic glutamate, or any other GPCR receptors known to couple via G proteins.

"Constitutive activation" as used herein means an agonist induced, polyphosphorylated receptor state where agonist is no longer required for continuous signal transduction. Because many disorders may be mediated by the imbalance of the ratio of agonist activatable receptors to constitutively activated receptors (designed herein as "GPCR/GPCR\*"), this ratio may be clinically manipulated in accordance with the invention for therapeutic applications.

One aspect of the present invention is to provide a screening assay for determining whether particular G protein coupled receptors are constitutively activatable by prior agonist exposure. This regulatory process is fundamentally different from the already known basal activity of many GPCRs.

Another aspect of the present invention is to provide a screening assay for classifying compounds as agonists, neutral antagonist, or negative antagonist, each having differential effects on signalling and phosphorylation of the G protein coupled receptor. Because this expanded categorization explicitly considers the effect of the constitutively activated, polyphosphorylated GPCRs, rather than the basal activity which results from spontaneous flipping on of unmodified receptors, it is more informative than the prior art classification of agonist and neutral and negative antagonist. Moreover, differential effects of ligand on several downstream events had not been considered previously.

Another aspect of the present invention is to provide a method for modulating the ratio of GPCR/GPCR\*.

In a preferred embodiment of the present invention, use of screening assays permits the identification of several agonists, neutral antagonists,

negative antagonists, and G protein kinase inhibitors capable of reducing the constitutively active  $\mu^*$  state of the  $\mu$  opioid receptor, as the driving force in the development of narcotic tolerance and dependence. An agent shown to prevent and reverse the conversion of  $\mu$  opioid receptors to the constitutively active  $\mu^*$  opioid receptors *in vitro*, has been shown to prevent and/or reverse narcotic tolerance and dependence in animal models.

Accordingly, practice of the invention is useful to determine or to screen for new pharmaceuticals useful for treating disease states mediated by G protein coupled receptors capable of constitutive activation, to enhance the clinical utility of existing pharmaceuticals targeted to G protein coupled receptors, such as via modulations of the GPCR/GPCR\* ratio, and to devise therapeutic treatments from agents selected by means such as the screening methods of the invention.

#### Detailed Description of the Preferred Embodiments

The principal novel tenet of the subject invention is that receptor phosphorylation can cause constitutive activation of some GPCRs, rather than desensitization. The phosphorylated receptor is thereby locked into the active state,  $R^*$ , as if occupied by an agonist, which is distinct from basal activity. A powerful positive feed-forward loop evolves, by which  $R^*$  is continuously signalling and simultaneously stimulates its own polyphosphorylation. As a result, the polyphosphorylated  $R^*$  can be kept in the active state for prolonged time periods, even though single phosphorylation and dephosphorylation steps are fast. Moreover, exposure to agonists enhances formation of  $R^*$  by further stimulating receptor phosphorylation. This kinetic trapping mechanism represents a novel, potent,

long-lasting positive regulatory mechanism with potentially profound and wide-ranging physiological implications. Any receptor undergoing this type of constitutive activation would be expected to contribute dominantly to the overall basal tone in the body, thereby modifying tonicity caused by continuously released hormones and neurotransmitters.

The invention generally relates to the discovery of agonist induced conversion of G protein coupled receptors to a constitutively active, polyphosphorylated state, R\*. In certain G protein receptors, an agonist induces signal transduction and simultaneously induces self-phosphorylation by G protein receptor kinases which are selective for the activated form of the receptor. While phosphorylation generally leads to receptor desensitization, phosphorylation of this class of G protein coupled receptors leads to constitutive activation. Independent of receptor agonist, constitutively activated GPCRs can transduce signal for a prolonged period of time.

The basis for any assay for constitutive activation of G protein coupled receptors is receptor phosphorylation and/or the activity of the constitutively active G protein coupled receptors. A non-obvious key element of such an assay is to pretreat the receptor preparation with an agonist, followed by complete removal of the agonist, and subsequently to measure constitutive activity with the use of neutral and negative antagonists.

One simple assay is to measure the constitutive activity of the G protein coupled receptor after pretreatment with (a) agonist alone, (b) agonist and G protein receptor kinase inhibitor to inhibit R\* formation, either in the absence or presence of a neutral or negative antagonist. Because constitutive



activation is mediated by phosphorylation, phosphatase inhibitors should enhance constitutive activation and G protein receptor kinase inhibitors should suppress constitutive activation, if the G protein coupled receptor indeed has constitutive activity. A variation of this assay is to measure the direct phosphorylation of the G protein coupled receptor instead of the activity of the G protein coupled receptors. If negative and neutral antagonist are unknown, screens of known receptor antagonists are required to identify prototypal agents with neutral and negative activity against R\*.

Once a G protein coupled receptor is found to be regulated by constitutive activity, assay systems may be set up to screen for compounds with the desired selective properties. For instance, an agonist may be found that activates the GPCR without also inducing receptor phosphorylation. Such an agonist would prevent the formation of GPCR\*.

Morphine is an example of an agonist which induces signal transduction in addition to inducing receptor phosphorylation, which can account for its tolerance and dependence liability. Moreover, classical antagonists with respect to the GPCR in its ground state (ligands that bind to GPCR with no activity) can be additionally categorized for their simultaneous effects on the constitutively activated GPCRs\* (notwithstanding previous classifications of neutral and negative antagonists with regard to basal receptor activity which did not consider receptor polyphosphorylation).

If it has no effect on GPCRs\*, then the antagonist is classified as a neutral antagonist. If it blocks the activity of GPCRs\*, then the antagonist is classified as a negative antagonist (or inverse agonist). Moreover, these antagonists can also be

tested for their ability to suppress continued GPCR\* phosphorylation.

Among the G protein coupled receptors that are capable of constitutive activation are the opioid receptors. For example, continuous stimulation of the  $\mu$  opioid receptor with morphine results in enhanced conversion to the constitutively active state  $\mu^*$ , as a principal new mechanism underlying the tolerant-dependent state. Thus, the  $\mu$  opioid receptor system serves as an example for identifying novel agents that affect formation of the constitutively active receptor state and any resultant long-term effects, such as tolerance and dependence to narcotic agonists.

Naloxone is an example of a negative antagonist which blocks constitutive signalling of  $\mu^*$ , but does not suppress or even enhances continuous  $\mu^*$  phosphorylation, as determined by the assays described here. Therefore, naloxone causes immediate and severe withdrawal in the dependent state, but it fails to reverse the dependent state which continues beyond the duration of naloxone in the body. Therefore, the proposed assays would predict such *in vivo* properties of antagonist and permit the selection of antagonists that could actively reverse the dependent state by suppressing continuous  $\mu^*$  phosphorylation.

Thus, the GPCR/GPCR\* ratio can be manipulated by any combination of full agonist, neutral agonist, neutral antagonist, negative antagonist, GRK kinase inhibitor, or phosphatase inhibitor. If an imbalance of GPCR/GPCR\* causes a certain disease state, the present invention provides the methods for modulating the GPCR/GPCR\* ratio to treating that disease state.

Although the invention will be specifically described using  $\mu$  opioid receptors, it may be generalized to any G protein coupled receptor that is

capable of constitutive activation upon stimulation with an agonist. G protein coupled receptors that are within the scope of this invention include all subtypes of the opioid, muscarinic, dopamine, adrenergic, cAMP, opsins, angiotensin, serotonin, thyrotropin, gonadotropin, substance-K, substance-P and substance-R, melanocortin, metabotropic glutamate, vasoactive intestinal peptide, secretin, and any other GPCR receptors.

In describing practice of this invention, a source of opioid  $\mu$  receptors in combination with a means of monitoring constitutively active  $\mu^*$  receptors, such as G protein activity (GTPase activity or GTP exchange) or the cAMP second messenger system, will together sometimes hereinafter be termed the "biological system." One source of opioid  $\mu$  receptors that are exposed to or coupled with cAMP production is a human neuroblastoma (NB) cell line (SK-N-SH) and its NB subclone SH-SY5Y, both which express abundant opioid  $\mu$  receptors (about 50,000 sites per cell). When intact cells are grown under appropriate cell culture conditions, the cells will be producing cAMP. Another source of a useful biological system for purposes of this invention can be certain tissues from experimental animals (e.g. rats and mice, which are good models for opioid  $\mu$  receptor activity in humans), such as rat locus coeruleus or guinea pig ileum.

When whole cells are used as the biological system, then it is desirable to add an adjuvant or stimulating agent of adenylyl cyclase, such as PGE, VIP, or forskolin, which stimulate cAMP production and therefore facilitate assay of the inhibitory effect of the  $\mu$  receptor. The phosphodiesterase inhibitor IBMX is frequently added to further enhance cAMP levels; however, as shown below, IBMX was determined by the assays in this application to be a  $\mu$  receptor kinase

inhibitor (and hence a prototype of a new class of potential anti-addictive agents); therefore, it must be avoided in the assay. Neuroblastoma cells are preferably first differentiated with, for example, 1-10  $\mu$  M retinoic acid to enhance stimulatory and inhibitory receptor coupling to the cAMP system. Such preparations of a biological system have been described by Yu et al., *J. Neurochem.*, 51, pp. 1892-1899 (1988); Yu et al., *J. Neurochem.*, 55, pp. 1390-1396 (1990); and Yu and Sadée, *J. Pharmac. Exp. Ther.*, 245, pp. 350-355 (1988).

A particularly preferred source of opioid  $\mu$  receptors that are exposed to or coupled with cAMP production is the HEK293 cell line stably transfected with the  $\mu$  receptor gene. However, any cell line that expresses naturally occurring  $\mu$  opioid receptors or cloned  $\mu$  opioid receptors will work. When intact cells are grown under appropriate cell culture conditions, the cells will be producing cAMP. Another source of  $\mu$  opioid receptors are certain tissues from animals such as rat locus coeruleus or guinea pig ileum.

In the opioid system, binding of a agonist (such as morphine) to the  $\mu$  opioid receptor leads to the inhibition of adenylyl cyclase which ultimately results in decreased levels of cAMP. Upon prolonged agonist exposure, the cAMP system is unregulated to compensate for the agonist induced inhibition. Ultimately, the effects of the increase numbers of  $\mu^*$  receptors and the unregulated cAMP cancel each other out, at least partially. Consequently, a relatively small spontaneous overshoot of cAMP is observed in an agonist exposed receptor system upon the removal of the agonist (hereinafter referred to as the "spontaneous overshoot"). This spontaneous overshoot of cAMP is the difference between the cAMP levels of a dependent receptor system upon the removal of an agonist and the

control levels of cAMP in the absence of any agonist, and is one of the accepted biological markers for narcotic dependence. In order to detect constitutive  $\mu^*$  activity continuously suppressing cAMP production even after agonist removal, one adds a negative antagonist, such as naloxone (its negative character being defined by the present assays). By suppressing  $\mu^*$  inhibitory signalling, naloxone causes an additional cAMP overshoot, referred to here as the naloxone cAMP overshoot, indicative of  $\mu^*$  and the dependent state.

Using cAMP as a surrogate measure for receptor activity, one assay embodiment in accordance with the invention involves the measurement of three values. A first cAMP value is determined by measuring the effects of a first portion of receptors on cAMP production in the absence of agonist pretreatment. This first cAMP value acts as a control value. Second and third cAMP values are also determined after agonist pretreatment and agonist removal. The second cAMP value is determined by measuring the effects of a second portion of receptors on cAMP production while the receptors are in a constitutively active state but are substantially free of agonist molecules. The difference between the second cAMP value and the first cAMP value represents the upregulated activity of the cAMP second messenger system, resulting from agonist pretreatment, which is however partially suppressed by  $\mu^*$  activity. Presence of such constitutive  $\mu^*$  activity represents a novel aspect which had not been suspected previously. The third cAMP value is determined by measuring the effects of a third portion of the receptors on cAMP production while they are in a constitutively active state, are substantially free of any agonist molecules, and are in the presence of a sufficient quantity of a negative antagonist to associate negative antagonist molecules

with substantially all the receptors. The difference between the third cAMP value and the second cAMP value represents the activity of the constitutively active  $\mu^*$  opioid receptors.

5 By "substantially free" of agonist molecules is meant that there is less than about 0.1% to about 0.3% of the total agonist drug remaining after pretreatment with a near maximally effective dose so  
10 that there would be no measurable effect in response curves. One can make removal determinations through use of radioactively labeled agonist tracer, or radioimmune assays, or one can analyze the wash water for residual agonist by using it to expose naive cells and  
15 determining whether there is an agonist effect. Typically, by washing cells carefully three times, substantial agonist removal is accomplished. If one is performing the assay *in vivo*, then the tissue is removed, sliced, and is washed in a water bath.

Because the conversion of  $\mu$  to  $\mu^*$  activity is  
20 mediated by phosphorylation, kinase inhibitors should prevent receptor phosphorylation while phosphatase inhibitors should enhance this process. As predicted, these results have been observed *in vitro* systems. For example, the general kinase inhibitor H7 completely  
25 prevents the formation of  $\mu^*$  receptors and rapidly reverses  $\mu^*$  activity in SH-SY5Y cells. As a result, co-administration of kinase inhibitors with existing analgesics should permit the use of these analgesics without the development of tolerance and dependence.  
30 Alternatively, kinase inhibitors should serve as effective agents for treating narcotic addiction.

Suitable G protein receptor kinases for practicing the invention in addition to the general kinase inhibitor H7, include the xanthine analog, 3-  
35 isobutyl-1-methyl-xanthine (IBMX). Although generally

recognized and used as CAMP phosphodiesterase inhibitor (Beavo et al., *Molec. Pharmacol.*, 6, 597 (1971)), IBMX is recognized here for the first time as a  $\mu$  receptor kinase inhibitor which suppresses  $\mu^*$  formation. Thus, 5 IBMX represents a prototypal agent of a class of drug congeners with broad pharmacological implications, including caffeine, theophyllin, theobromine, etc. This invention now permits the selection of alkylated xanthines and congeners with preferential activity 10 against receptor kinases, rather than CAMP phosphodiesterases. Such inhibitors may function as general GPCR kinase inhibitors, and therefore, may also be applicable to other GPCRs that are activatable by GPCR kinases, e.g., as proposed for the dopamine 15 receptor. For example, in a manner analogous to the coadministration of a kinase inhibitor with a narcotic analgesic to prevent or to retard the development of tolerance, the coadministration of a GPCR kinase with L-dopa is believed useful in treating Parkinsonism. 20 Moreover, in disease states characterized by hyperactivity of dopamine neurotransmission, administration of a GPCR kinase inhibitor is thought to reduce the dopamine receptor hyperactivity, and thereby, alleviate disease symptoms. Alternatively, such kinase inhibitors 25 could also serve to suppress desensitization during drug agonist treatment, for example of the  $\beta_2$  adrenergic receptor, which is thought to desensitize upon phosphorylation.

Finally, direct measurements of  $\mu$  receptor 30 phosphorylation can be used. Using the cloned  $\mu$  receptor gene transfected into HEK293 cells, phosphorylation of the  $\mu$  receptors can be directly measured. As predicted by the proposed mechanism, the  $\mu$  receptor is continuously phosphorylated by  $\mu$  receptor 35 kinases (GRKs) because it already preexists in part in

the polyphosphorylated  $\mu^*$  state and rapidly exchanges phosphate. Moreover, the addition of kinase inhibitor H7, and of IBMX, inhibited  $\mu^*$  receptor phosphorylation. Consequently, the direct measurement of  $\mu$  receptor phosphorylation can also serve as another screen for compounds that can manipulate the  $\mu/\mu^*$  ratio.

Suitable phosphatase inhibitors for practicing the present invention include calyculin-A. Since the PPase-2 selective okadaic acid at a low concentration (15 nM) is ineffective in enhancing  $\mu^*$ , it appears that PPase-1 is responsible for dephosphorylating  $\mu^*$  in SH-SY5Y cells. However, phosphatases may vary in different tissues, and with different receptors.

Accordingly, an inventive assay system can be readily set up to classify compounds for their effects on G protein coupled receptors, such as on the  $\mu$  opioid receptors. Using any one or a combination of the three markers, CAMP (second messenger system), receptor phosphorylation and G protein receptor kinase activity, compounds may be classified (as agonist, neutral antagonist, negative antagonist) for their effects both on the agonist activatable  $\mu$  receptors and the constitutively active  $\mu^*$  receptors. For a more general assay of GPCR signalling, instead of CAMP assays, one can substitute assays of G protein activity, such as GTPase activity or GTP exchange, which represent the more proximal receptor signalling events and are generally applicable to all GPCRs, regardless of their second messenger system.

Using such an assay, compounds may be screened for their selective effects on either the  $\mu$  or  $\mu^*$  receptors. For instance, agonist for the  $\mu$  opioid receptors may be found that do not also have the ability to induce or limit  $\mu$  opioid receptor phosphorylation. Such an agonist would not induce narcotic tolerance or



dependence and would thus be an excellent analgesic for treating patients with chronic pain. Similarly, the assay can also be used to screen for compounds that can be used in conjunction with known full agonists (such as morphine) to prevent the development of tolerance and dependence by inhibiting the formation of the constitutively active  $\mu^*$  receptors. These compounds may include GPCR kinase inhibitors to prevent receptor phosphorylation.

If the goal is to search for compounds to treat a narcotic dependent individual, then the same assays may be used to screen for neutral antagonists that will bind to the agonist activatable  $\mu$  opioid receptor to prevent agonist action while at the same time not eliciting the symptoms of withdrawal by blocking the activity of the constitutively active  $\mu^*$  opioid receptors. Thus, compounds can be determined that have the properties of blocking the effects of a narcotic analgesic such as morphine or of both morphine and naloxone with no effect when given alone, so as to be considered useful for treating overdoses of narcotic analgesics while avoiding the risk of excessive precipitated withdrawal. This class of compounds should be useful to prevent or reverse the generation of constitutively active receptors and thus can be used therapeutically in conjunction with a narcotic analgesic to suppress the addictive liability of the narcotic analgesic.

Thus, a therapeutic method for treating a patient addicted to a narcotic drug is provided since one can use assays of the invention to select an agent determined to prevent and/or to reverse constitutive activity of opioid receptors and then administer a therapeutically effective amount of the selected agent to the addicted patient. Further, one may treat

patients addicted to a narcotic drug, who are suffering from an overdose (or suspected overdose) of the narcotic drug by selecting a neutral  $\mu$  receptor antagonist that does not block  $\mu^*$  activity. Preferably, the agent  
5 selected would also reverse the  $\mu^*$  state to the normal  $\mu$  receptor state. In another aspect, a therapeutic method for providing analgesia to a patient can comprise selecting a  $\mu$  receptor agonist that results in lower conversion of the  $\mu$  receptor to its constitutively  
10 active state than does a narcotic analgesic such as morphine, and administering such  $\mu$  receptor agonist for the treatment for pain, with the desirable properties of reduced tolerance and dependence. Yet further, a therapeutic method for providing analgesia to a patient  
15 is by selecting a kinase inhibitor and administering a narcotic analgesic in conjunction with the selected kinase inhibitor. The kinase inhibitor selected is effective to retard development of tolerance to the narcotic analgesic administered, such as for preventing  
20 the formation of constitutively active opioid receptors.

Because the process of constitutive activation lends itself to screening anti-addictive agents and probing the molecular mechanisms of narcotic dependence, practice of the invention is expected to provide a new  
25 approach to separating the beneficial activity of narcotics from undesirable long-term effects.

Pharmaceutically effective amounts of agents selected (as by the screening method herein discussed) may be readily determinable clinically by establishing  
30 safe dosages and dose-response curves, such as in established clinical pain models. For example, analgesia in rodent animal models can be measured by the tail-flick method of D'Amour and Smith, *J. Pharmac. Exp. Ther.*, 72, pp. 74-79 (1941), and as modified by Tulunay  
35 and Takemori, *J. Pharmac. Exp. Ther.*, 190, pp. 395-400

(1974), both incorporated herein by reference. ED<sub>50</sub> values, their 95% confidence limits, and significance of potency relation between two ED<sub>50</sub> values may be determined by the method of Litchfield and Wilcoxon, *J. Pharmac. Exp. Ther.*, 96, pp. 99-113 (1949), incorporated herein by reference.

By "narcotic analgesic" as used herein and exemplified by morphine, is meant in addition to morphine, the morphine salts (such as morphine hydrobromide, morphine hydrochloride, morphine mucate, morphine oleate, morphine N-oxide, and morphine sulfate), and morphine analogs such as normorphine, diacetyldihydromorphine, diacetylmorphine hydrochloride, codeine, and diacetylmorphine (heroin). Other widely used narcotic analgesics with which the present invention may be used include alphatrodine, methadone, merperidine, leverthanol, propoxyphene, fentanyl, oxymorphone, anileridine, and metopon.

Administration of the selected agent in conjunction with administering a dose of a narcotic analgesic can be within at least about 30 minutes of the narcotic analgesic dose. Preferably, the administering is by administering a single, admixed dose where the narcotic analgesic is morphine, a morphine analog, or a morphine salt. Thus, administrations may be intravenous and formulations of pharmaceutically acceptable solutions, carriers, or salts as are well known to the art may be used. Depending upon the agent selected, other forms of administration may be found to be clinically useful.

Moreover, antagonists may be found that block continuous  $\mu^*$  phosphorylation. G protein receptor kinase inhibitors may also be included in the treatment regimen to prevent any further conversion of  $\mu$  to  $\mu^*$  receptors.

In summary, any combination of agonist, neutral antagonist, negative antagonist, GPCR kinase inhibitors, and phosphatase inhibitors may be used to manipulate the  $\mu/\mu^*$  ratio. Because the development of narcotic tolerance and dependence is characterized by the slow conversion of the  $\mu$  to  $\mu^*$  receptors, most of the compounds for therapeutic benefit are focused on the prevention of the formation of the  $\mu^*$  receptors. However, this focus is only with respect to the  $\mu$  opioid receptors. In other G protein coupled receptors, it may be desirable to form GPCR\* in order to maintain the GPCR/GPCR\* at the optimal level. Depending on whether the disease state is mediated by the abundance of one receptor state versus the other, different combinations of agonist, neutral antagonist, negative antagonist, GRK kinase inhibitors, and phosphatase inhibitors will be used. Since the present invention discloses the general mechanism for these G protein coupled receptors, persons skilled in the art will be able to ascertain which combinations of agonist, neutral antagonist, negative antagonist, GRK inhibitors, and phosphatase inhibitors to use to achieve the desired ratio of GPCR/GPCR\*.

It is to be understood that the description and the following examples are intended to illustrate and not limit the scope of the invention.

#### EXAMPLE 1

##### Regulation of cAMP in Cell Culture

Although the basic assay will be described using SK-N-SH or SH-SY5Y cells, it is readily adaptable to other cell lines or tissue samples as the useful biological systems. The cells were grown at 37°C in DME H-21 medium supplemented with 10% fetal calf serum

containing 100  $\mu\text{g/ml}$  streptomycin and 100 IU/ml penicillin. Optimal sensitivity to  $\mu$  opioid agonist inhibition of cAMP accumulation was observed in retinoic acid pretreated cells (5  $\mu\text{M}$  for 6 days), and upon  
5 adenylyl cyclase stimulation with prostaglandin  $\text{E}_1$  ( $\text{PGE}_1$ ), in the absence of any phosphodiesterase inhibitor.

To induce a tolerant-dependent state, the cells were pretreated with 1  $\mu\text{M}$  morphine for period  
10 ranging from 2 minutes to 48 hours. The relatively low pretreatment concentration of 1  $\mu\text{M}$  morphine was selected to achieve near maximal effects in tolerant cells while facilitating complete washout of the drug. Immediately before the cAMP accumulation assay, cells  
15 were washed twice with medium containing 5% serum and twice more with serum free medium. Supernatants from the washed cells failed to elicit any opioid agonist like cAMP response when transferred to fresh untreated cells, suggesting effective morphine removal during  
20 washing. Use of  $^3\text{H}$ -morphine also indicated that 0.1% of the pretreatment concentration was left in the final assay medium, which was below effective morphine levels in this cell line. The values for cAMP levels were determined by radioimmunoassay after stimulation with 1  
25  $\mu\text{M}$  prostaglandin  $\text{E}_1$  over 15 minutes in the presence or absence of naloxone.

After pretreatment of SH-SY5Y cells for 6 to 12 hours with morphine, and complete drug removal by thorough washing of the cells, a spontaneous cAMP  
30 overshoot was observed when compared to untreated cells. Moreover, addition of naloxone to the pretreated and thoroughly washed, drug-free cells caused a significant additional increase of cAMP accumulation in proportion to the level of tolerance/dependence developed by the  
35 cells (this naloxone induced cAMP overshoot ranged from

20 to 80% in over 40 experiments,  $p < 0.001$ ). The difference between the naloxone induced CAMP overshoot and the spontaneous CAMP overshoot represents the activity of the  $\mu^*$  receptors in suppressing CAMP levels that is now blocked by the negative antagonist naloxone. Therefore, the naloxone CAMP overshoot provides a novel measure of constitutive receptors' activity observed after agonist pretreatment.

The naloxone CAMP overshoot was also observed in extensively washed membrane homogenates obtained from morphine pretreated cells, further arguing against residual morphine as a cause. Moreover, results with neutral antagonists CTAP and CTOP which do not affect CAMP levels (described below) rule out residual opioids residing at the receptor as the cause for the naloxone CAMP overshoot. When naloxone was applied to thoroughly washed, untreated SH-SY5Y control cells, a decrease of CAMP accumulation was observed. The decreased CAMP levels relative to control levels by an antagonist indicates presence of some constitutive activity in untreated cells which is in equilibrium with the agonist activatable  $\mu$  receptors. Such small basal receptor activity paradoxically enhances CAMP production in this case. Paradoxical stimulation of CAMP production by GPCRs normally thought to be inhibitory have been observed repeatedly with varying receptors and cell culture conditions. In the case of the  $\mu$  receptor, such changes from stimulatory to inhibitory actions (in response to agonists) have been proposed to contribute to narcotic tolerance and dependence.

To account for tolerance and dependence, the spontaneous and the naloxone CAMP overshoot should occur gradually. Pretreatment of cells with 1  $\mu$ M morphine for 20 minutes or less caused a small decrease of PGE<sub>1</sub> stimulated CAMP accumulation, which was not reversed or

even enhanced by the addition of naloxone. Therefore, this decrease could not have resulted from residual morphine. Morphine pretreatment longer than 20 minutes caused gradually increasing levels of both the spontaneous and the naloxone cAMP overshoot, until a maximum is reached at 12 hours. A treatment period of 12 hours for maximal cAMP overshoot is compatible with the slow development of tolerance and dependence.

The spontaneous cAMP overshoot disappeared within 30 minutes after morphine removal. This result supports the hypothesis that the spontaneous cAMP overshoot is not responsible for the prolonged dependent state. In contrast, the naloxone cAMP overshoot lasted for at least two hours. From 4-12 hours after morphine removal, naloxone had no effect on cAMP levels, in contrast to the decrease in cAMP levels observed in untreated cells. This observation is consistent with the delayed peak of narcotic withdrawal seen *in vivo*, which occurs after morphine is largely eliminated from the body.

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Because many G protein coupled receptors are also linked to the signal pathway via cAMP, the differential ligand effects on cAMP levels can be readily adapted for any particular G protein coupled receptor. As described above, any cells that are capable of producing cAMP under cell growth conditions (the useful biological system) and that abundantly express either naturally occurring G protein coupled receptors or cloned G protein coupled receptors may be used. Another major second messenger pathway involves turnover of phosphatidyl inositol (PI), e.g., for the muscarinic m1 receptor. The activity of all GPCRs can be assessed by measuring GTPase activity and GTP exchange.

One variation of the assay involves the identification of a negative antagonist as described above. The most efficient method for finding negative antagonists would be to screen known antagonists of the particular G protein coupled receptors for negative  
5 intrinsic activity. Antagonists may be identified using standard competition assays that are well known in the art. If the binding of an agonist to the G protein coupled receptors results in increasing the levels of  
10 cAMP relative to control levels of cAMP, then compounds that decrease the levels of cAMP relative to control levels of cAMP in the absence of agonist would indicate negative intrinsic activity. If the binding of an  
15 agonist to the G protein coupled receptors results in decreasing the levels of cAMP relative to control levels of cAMP, then compounds that increase the levels of cAMP relative to control levels of cAMP in the absence of agonist would indicate negative intrinsic activity.

With respect to  $\mu$  opioid receptors, the basic  
20 assay described in Example 1 can be readily adapted to classify compounds as agonist, negative antagonist, and neutral antagonist depending on their effects on the levels of cAMP.

A full agonist is an agonist that activates  
25 signal transduction and induces receptor phosphorylation leading to a slow conversion of  $\mu$  to  $\mu^*$  receptors upon prolonged exposure. Using the cAMP levels, a full agonist would inhibit cAMP levels and would induce a naloxone induced cAMP overshoot in the dependent state.

30 In contrast, a novel type of agonist activates only signal transduction but would not induce receptor phosphorylation. Because there would be no slow conversion from  $\mu$  to  $\mu^*$  receptors, the naloxone cAMP overshoot would not be observed if naloxone is added to  
35 an agonist exposed system in the absence of agonist.



Although ideally, such an agonist would be optimal, any agonist that slows the formation of  $\mu^*$  receptors relative to such existing analgesics like morphine would also be a therapeutically useful.

5                   Antagonists for the  $\mu$  opioid receptors may also be additionally classified as either neutral antagonist or negative antagonist depending on their effect on the constitutively activated  $\mu^*$  opioid receptors. A neutral antagonist is a classical  
10 antagonist with respect to the agonist activatable  $\mu$  opioid receptors and does not block the signalling activity of the constitutively active  $\mu^*$  opioid receptors. In contrast, a negative antagonist is a classical antagonist with respect to the agonist  
15 activatable  $\mu$  opioid receptors and blocks the signalling activity of the constitutively active  $\mu^*$  opioid receptors. Moreover, neutral and negative antagonists can be classified as to their effect on  $\mu^*$  phosphorylation. For example, while naloxone is a  
20 negative antagonist with respect to signalling, it failed to suppress  $\mu^*$  phosphorylation. Therefore, it produces withdrawal, but does not reverse the dependent state.

                  The  $\mu$  selective agonist peptide DAMGO was  
25 also tested for its ability to induce signalling and receptor phosphorylation in  $\mu$  and  $EE\mu$  receptor transfected HEK293 cells. DAMGO was slightly more potent and efficacious than morphine in both activity tests, when added acutely to the assay incubations.  
30 However, in a separate experiment, it was shown that DAMGO, but not morphine induced massive receptor recycling through endocytic vesicles. Since recycling of the  $\beta_2$  receptor was associated with dephosphorylation, it is possible that during long-term  
35 exposure to an agonist such as DAMGO, overall  $\mu^*$

formation is lower than with morphine. While this remains to be tested, the distinct downstream effects of morphine and DAMGO demonstrate that different agonists do affect different downstream pathway even at the same receptor.

The ability of the neutral antagonist CTAP to affect  $\mu^*$  phosphorylation during the assay incubation was also tested. While CTAP did not block  $\mu^*$  phosphorylation, it consistently lowered the level of  $\mu^*$  phosphorylation relative to naloxone. This result suggests that CTAP may affect the balance of  $\mu$  and  $\mu^*$  receptors in favor of the ground state  $\mu$ , and therefore, it is potentially useful not only in the treatment of narcotic overdose in addicts, but also as an agent capable of at least partially reversing the tolerant-dependent state.

Classification as either neutral antagonist or negative antagonist can be determined by the addition of the compound to a agonist-free but narcotic dependent cell system (as in Example 1) that has been pretreated with morphine for at least 12 hours. If there is no additional cAMP overshoot above the spontaneous cAMP overshoot, then the compound is a neutral antagonist. If there is an additional cAMP overshoot above the spontaneous cAMP overshoot, then the compound is a negative antagonist.

## EXAMPLE 2

### **Screening for Compounds Using cAMP Levels as a Marker**

Using the assay of Example 1, a panel of opioid drugs were classified for their ability to either decrease or increase cAMP levels in morphine pretreated SH-SY5Y cells. As expected from their high potency in

causing withdrawal, naloxone, naltrexone, and diprenorphine demonstrated negative intrinsic activity by increasing cAMP levels in drug-free dependent cells. Consequently, naloxone, naltrexone, and diprenorphine  
5 were classified as negative antagonists. Buprenorphine, DAMGO and DADLE were classified as full agonists and are either as effective if not more so than morphine. Nalorphine, CTAP and its analogs, CTOP and d-Tic-CTAP were classified as neutral antagonists because they had  
10 no significant effect on cAMP levels in dependent cells.

---

Similarly, the cAMP levels could be used to classify compounds with respect to any particular G protein coupled receptor that transduces signal via the cAMP pathway. If the binding of an agonist to the G  
15 protein coupled receptors results in increasing the levels of cAMP relative to control levels of cAMP, then an antagonist that decrease the levels of cAMP relative to control levels of cAMP in the absence of agonist would be a negative antagonist. If the binding of an  
20 agonist to the G protein coupled receptors results in decreasing the levels of cAMP relative to control levels of cAMP, then an antagonist that increase the levels of cAMP relative to control levels of cAMP in the absence of agonist would be a negative antagonist. If the  
25 antagonist had no effect on the levels of cAMP relative to control levels, then the antagonist would be a neutral antagonist.

Alternatively, measurement of PI turnover could replace cAMP assays where appropriate (ml  
30 receptor). Lastly, direct assays of G protein activity (GTPase and GTP exchange) are universally applicable to all GPCRs. Negative antagonists would suppress, and agonists would enhance G protein activity, while neutral antagonists would be without effect.

A full agonist with respect to a particular G protein coupled receptor is an agonist that also induces receptor phosphorylation and the resulting slow conversion of GPCR to GPCR\*. This may be established by monitoring the levels of cAMP after prolonged exposures to the agonist. The cAMP levels are measured relative to the levels after removal of agonist after prolonged exposure. If there is an additive change in the levels of cAMP when a negative antagonist is added, then the agonist is a full agonist. If there is no additional change or even a subtractive change in the levels of cAMP when a negative antagonist is added, then the agonist is a neutral agonist. A subtractive effect might result if there are significant preexisting basal levels of GPCR\*. PI coupled receptors can be analyzed in an endogenous fashion, or arrays of G protein activities are used.

### EXAMPLE 3

Effects of H7 and CTAP  
in Morphine Tolerant Dependent Mice

#### Withdrawal

Male ICR mice in groups of 10 each were made acutely dependent on morphine by subcutaneous injection of 100 mg/kg morphine sulfate. After 4 hours, naloxone was given either intraperitoneally or simultaneously by intracerebroventricular and intrathecal injections to induce withdrawal. Alternatively, CTAP was injected. To test the ability of kinase inhibitors to reverse the dependent state, animals were injected 3.5 hours after the first 100 mg/kg morphine dose with saline or with H7 or H8. At 4 hours, withdrawal was precipitated by injection of naloxone (3 mg/kg intraperitoneally). Mice were then placed in Plexiglass cylinders and observed

for a 15 minute period with the number of vertical jumps recorded.

### Tolerance

Mice in groups of 10 were again injected subcutaneously with 100 mg/kg morphine sulfate, and analgesia was measured with the tail immersion assay at 55° C by determining the time elapsed before the tail is flicked (15 seconds cutoff = 100% analgesia). At 4.5 hours after the first dose, either saline alone or saline containing 50 nM H7 was injected. At 5 hours after the analgesic effect of first morphine dose had essentially ceased and a second dose of 10 mg/kg morphine sulfate was injected to determine the degree of tolerance.

### Results

The formation of  $\mu^*$  can account for narcotic dependence, and paradoxically also for tolerance. Assuming that all  $\mu$  receptors were converted to  $\mu^*$ , it follows that further addition of an agonist would be without effect, because all receptor are already active, and the organism is adapted to this activity. If  $\mu^*$  activity were responsible for narcotic tolerance and dependence, then the following effects in morphine pretreated animals would be predicted. First, H7 should reverse tolerance and suppress naloxone induced jumping without affecting morphine analgesia whereas H8 should be without any effect. Second, the neutral antagonist CTAP should cause less withdrawal than naloxone because it does not reverse the activity of  $\mu^*$ . Finally, CTAP should reverse naloxone induced jumping.

Administration of H7 30 minutes before naloxone strongly suppressed the number of naloxone precipitated jumps without affecting gross behavior of

the animals or morphine antinociception as measured in the 55° C hot water tail-flick test. In contrast, H8 was without effect on naloxone induced jumping. Therefore, the protein kinase inhibitor H7 reversed the drug dependent state towards the naive state within 30 minutes. H7 also fully reversed morphine tolerance observed 5 hours after a 100 mg/kg morphine sulfate dose in mice. The test dose of 10 mg/kg morphine sulfate produced only 22±14% analgesia in the tail flick assay when given 5 hours after the first dose, and 30 minutes after a control injection of saline. However, injection of 50 nM H7 30 minutes before the second morphine test dose completely reversed tolerance yielding 90±10% analgesia. Therefore, H7 reversed both morphine tolerance and dependence in an acute mouse model as predicted.

As expected for a neutral antagonist, CTAP caused significantly less withdrawal jumping, from 77±20 jumps for naloxone to 32±12 jumps, and it partially reversed naloxone induced withdrawal jumping. These combined results document that the predictions from the *in vitro* results can be verified in an animal model of narcotic tolerance and dependence. This is the first time that agents have been successfully used to reverse tolerance and dependence, selected on the basis of a novel mechanism of  $\mu$  receptor phosphorylation and constitutive activation.

#### EXAMPLE 4

Radiolabeled agonists tracers can alternatively be used to rapidly screen for agents with an ability to bind to the constitutively active GPCR\*. Agonists are expected to have low affinity to the constitutively active receptors and can serve as an

analytical tool to block the GPCR to allow the selective labeling of the constitutively active GPCR\*. The constitutively active GPCR\* are selectively labeled with radiolabeled neutral or negative antagonists, using  
5 agonist pretreated preparations to maximize  $\mu^*$ . Thus, when the receptors are exposed to a test composition, one is able to determine whether the test composition binds to the selectively labeled receptors. Those compounds that bind to the selectively labeled GPCR\*  
10 could then be further classified as a neutral antagonist or negative antagonist depending on their effects on the activity of GPCR\*. Because this assay is much faster than the cAMP type assays, it can be used as the first screen when testing a large number of compounds.

15

**EXAMPLE 5****Direct Measurement of  $\mu$  Receptor Phosphorylation****Epitope-tagged  $\mu$  opioid receptor (EE- $\mu$ R)**

The sequence, TTTTAAGCTT ACCATGGAAT ACATGCCAAT  
GGAAGACAGC AGCACCGGCC CAGGG (SEQ ID NO: 1), containing  
20 a *HindIII* restriction site, the start codon, and a sequence encoding the epitope EYMPME (underlined portion), served as the 5' primer in order to append the epitope tag to the amino terminal of the rat  $\mu$  receptor by the polymerase chain reaction. The 3' primer  
25 sequence, GCTCTAGAGC GAGGGTCTGG ATGGTG (SEQ ID NO: 2), contained a stop codon and an *XbaI* restriction site. The amplified fragment (EE- $\mu$ R) was ligated into pRc/CMV which contains a neomycin-resistance gene, and subcloned into the E. coli TOP 10F' strain. The 5' sequence  
30 containing the epitope was verified by automated sequencing. Human embryonic kidney cells (HEK293) were transfected by the calcium phosphate method, and clonal cell lines stably expressing EE- $\mu$ R were selected with

400 µg/ml G-418 and maintained in DMEM/H-16/F-12 with 10% fetal calf serum and 200 µg/ml G-418.

#### Ligand Binding Assays and cAMP assay

EE-µR expression was quantified with [3H]-  
5 diprenorphine. HEK293 cells stably expressing EE-µR  
were harvested in PBS, and triplicate samples were  
incubated with 10 nM [3H]-diprenorphine and differing  
concentrations of diprenorphine or morphine sulfate in  
50 mM Tris, pH 7.4. Cells were incubated for 2 hours at  
10 20° C and harvested on GF/C glass filters which were  
washed three times with cold PBS, and the tritium  
content determined. Displacement data were fit to the  
logistic function,  $B = B_{\max} - [B_{\min} * L] / [IC_{50} + L] + NSB$ ,  
where B is the tracer bound, L = diprenorphine or  
15 morphine concentration, and NSB is the nonspecific  
binding. Protein content was determined by the Bradford  
method. Bradford, *Anal. Biochem.*, 72, pp. 248-254  
(1976).

#### Immunoprecipitation of the Phosphorylated EE-µR

20 Phosphorylation of EE-µR was performed in  
permeabilized cells by a modification of the procedure  
described by Raymond. Raymond, *J. Biol. Chem.*, 266, pp.  
14747-14753 (1991). Confluent flasks with approximately  
2x10<sup>7</sup> HEK293 cells were washed gently in 50 mM Tris (pH  
25 7.4), 100 mM NaCl and incubated for 15 minutes at 37° C  
in phosphate-free DMEM. After harvesting and brief  
centrifugation cell pellets were resuspended in  
phosphate-free medium and aliquots of digitonic were  
added until approximately 90% of cells were  
30 permeabilized as defined by failure to exclude trypan  
blue dye. Final digitone concentration was 150-200 µM.  
Cells were treated with 10 µM morphine sulfate or  
buffer and 1 µCi/µl[γ-<sup>32</sup>P]-ATP in a final volume of 500



$\mu$ l for 15 minutes at 25° C with gentle rocking. Labeling medium was removed and cell pellets were washed twice in ice cold Tris-NaCl buffer containing phosphatase and protease inhibitors (50 mM Tris, pH 7.4, 100 mM NaCl, 10 mM sodium pyrophosphate, 10 mM NaF, 1 mM benzamidine, 1  $\mu$ g/ml leupeptin, and 1  $\mu$ g/ml aprotinin). Cells were resuspended in ice-cold lysis buffer containing phosphatase inhibitors, incubated on ice for 10 minutes, and homogenized in a Dounce homogenizer. A membrane pellet was prepared, solubilized in 10 mM CHAPS, and immunoprecipitated with 1:40 dilution of anti-EE monoclonal antibody. The incorporation of  $^{32}$ P into the supernatant was determined at the time of immunoprecipitation of equalized control and transfected samples. The 8% SDS-PAGE gels were autoradiographed and bands were quantified by scanning densitometry.

### Results

The epitope-tagged EEm receptor was similar to the wild-type  $\mu$  receptor in all pharmacological assays. Confirming predicted results, the epitope tagged  $\mu$  opioid receptor is significantly phosphorylated in the absence of any agonist, suggesting the presence of  $\mu^*$ . Agonist exposures (15 minutes) during the labeling assay increased the levels of phosphorylation of the receptors by nearly twofold. Moreover, pretreatment with 1  $\mu$ M morphine for 6-12 hours, followed by agonist removal before the phosphorylation assay, enhanced  $\mu$  receptor phosphorylation by threefold, indicative of agonist dependent conversion of  $\mu$  to  $\mu^*$ . Naloxone did not block  $\mu$  receptor phosphorylation, but in contrast appeared to slightly stimulate it. This result is consistent with the finding that naloxone does not reverse the dependent state *in vivo*. As predicted GRK kinase inhibitor H7 strongly inhibits  $\mu$  receptor

phosphorylation, either when added acutely to the assay, or via pretreatment and subsequent removal before the assay. This result suggest that  $\mu$  receptor kinase inhibition depletes the  $\mu^*$  state.

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5           As a result, assays may be modified to use  
direct receptor phosphorylation as a marker for  
classifying compounds as full agonist, neutral agonist,  
negative antagonist, neutral antagonist, and GOCR kinase  
10           inhibitor. Although these results are with respect to  
the  $\mu$  opioid receptors, they may be readily modified  
for any particular G protein coupled receptor.  
Moreover, G protein receptor kinase inhibitors may be  
found directly using standard enzyme activity assays.  
See generally, Chen et al., J. Biol. Chem., 268: 7825-  
15           7831 (1993).

          There are at least 7 genes encoding the family  
of related G protein receptor kinases that all have the  
same characteristics essential for supporting the  
positive feed-forward mechanism of sustained GPCR\*  
20           activity. These are expressed differentially in  
different tissues, providing the opportunity for  
selective effects; however, no selective inhibitors are  
available. This invention permits the selection of such  
kinase inhibitors and their evaluation as potential  
25           therapeutic agents.

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          Mental disorders involving the dopaminergic  
system include schizophrenia and Parkinsonism, which are  
associated with an excess and deficit, respectively of  
dopaminergic transmission. Moreover, dopaminergic  
30           pathways are thought to be essentially involved in  
reward mechanisms and drug cravings. However, the  
mechanisms underlying these processes are not  
understood.

As with the  $\mu$  opioid receptors which involve narcotic tolerance and dependence, an imbalance of the agonist activatable dopamine receptor (DR) and constitutively active dopamine receptors (DR\*) may be involved in schizophrenia, Parkinsonism, and drug cravings. Excess constitutively active dopamine receptor activity could be involved in schizophrenia, and drug seeking behavior/addiction. As an example, constitutive activation of the dopamine receptors could also explain why treatment of Parkinsonism with L-DOPA (which gets converted to dopamine) loses its effectiveness. Thus, in a manner analogous to the coadministration of a kinase inhibitor with a narcotic analgesic to prevent or to retard the development of tolerance, the coadministration of a GPCR kinase with L-dopa is believed useful in treating Parkinsonism, to prevent or retard the constitutively active state of dopamine receptors. Therefore, analogous to the results with the  $\mu$  opioid receptors, manipulating the DR/DR\* ratio could lead to treatment of schizophrenia and drug addiction, and to enhance existing therapies for Parkinson's disease.

Among the known dopamine receptor subtypes, the D2 receptor plays a prominent role in schizophrenia. Changes in D2 ligand binding characteristics that were observed in post-mortem CNS tissue from schizophrenic patients, have suggested that altered receptor-G protein coupling may play a role. Preliminary experiments analogous to those initially performed for the  $\mu$  opioid receptors in stably transfected HEK293 cells, indicate that D2 receptors are also regulated through constitutive activation. While quinpirole served as the agonist (equivalent to morphine), haloperidol, spiperone, and sulpiride all displayed negative antagonism. Spontaneous and negative antagonist induced

cAMP overshoots were observed after quinpirole pretreatment and thorough drug removal by washing. This result supports the view that the D2 receptor is also converted to a constitutively active state during  
5 agonist exposure. To determine D2 receptor phosphorylation, the same EE epitope tag was introduced into the D21 gene (long splice isoform), immediately adjacent to the ATG initiation codon. Thus, a similar set of assays can now be performed with the D21 receptor.

10

EXAMPLE 6

Using HEK293 cells stably transfected with the long splice isoform of the D2 receptor, incubation with a known antagonist, spiperone in the absence of agonist, resulted in decreased cAMP levels relative to control  
15 indicating intrinsic negative activity, as proposed for the  $\mu$  receptor. The effects of the agonist quinpirole and antagonist spiperone on cAMP levels before and after pretreatment with quinpirole were nearly identical to that of morphine and naloxone in the  $\mu$  opioid receptor  
20 system. Because the D2 receptor also transduces signal via the cAMP pathway, assays described for the  $\mu$  opioid receptor system may thus be readily adapted for the D2 receptor.

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As cholinergic pathways intimately associated  
25 with cognitive functions, cholinergic deficits are a hallmark of neurodegenerative disorders involved with memory impairment. Elevation in levels of acetylcholine are thought to enhance cognitive functions by postsynaptic action on the muscarinic m1 receptor. The  
30 muscarinic m1 receptor is coupled to phospholipase C which in turn stimulates phosphatidyl inositol (PI) turnover. One example of the attempt to modulate levels

of acetylcholine for therapeutic benefit is tacrine, an acetylcholinesterase inhibitor, for the treatment of Alzheimer's disease.

#### EXAMPLE 7

5           An extensive study of the m1 receptor which includes nearly 100 mutations of the m1 receptor gene has been undertaken in an effort to understand m1 signal transduction and regulation. One mutant, m1-E360A, was found to be a partially activated receptor and was  
10 stably transfected into HEK293 cells for further studies. Because of its partial activity, m1-E360A in HEK293 cells has a basal activity for stimulating PI turnover significantly above background.

          The availability of this mutant form of the m1  
15 receptor facilitated finding negative antagonists. Because of their decreasing effects on m1-E360A basal activity, atropine and scopolamine were found to be negative antagonists. As expected if constitutive activity were due to phosphorylation, pretreatment with  
20 agonist carbochol and with the phosphatase inhibitor calyculin A enhanced the basal activity. Therefore, HEK293 cells transfected with the m1 wild-type or the activating mutant E360A can serve in the assays equivalent to those described for the  $\mu$  receptor.

25

#### EXAMPLE 8

          Depending on the particular condition to be treated, it will be desirable to either increase or decrease the formation of the constitutively active GPCR\*. In either case, any combination of agonist,  
30 neutral antagonist, negative antagonist, GRK kinase inhibitor, and phosphatase inhibitor may be used to

obtained the desired effect. The classification of the compounds as agonist, neutral antagonist, negative antagonist, each with respect to signalling and phosphorylation, GPCR kinase inhibitor, or phosphatase inhibitor, can be achieved by the methods discussed in prior examples. For example, practice of the present invention may yield agonists that would transduce the desired signal but without the resulting constitutive activation of the receptors. If known full agonists are to be used that stimulate both signalling and phosphorylation, they may be used in combination with GPCR kinase inhibitor to prevent the formation of the constitutively active receptors. If attenuation of constitutive activity is sought to be treated, neutral antagonist and/or GPCR kinase inhibitor may be used. However, if the goal is to form constitutively active GPCR\*, then full agonist and/or phosphatase inhibitors may be used. Because the present invention discloses the general mechanism for these G protein coupled receptors, persons skilled in the art would know which combination of agonist, neutral antagonist, negative antagonist, GPCR kinase inhibitor, or phosphatase inhibitor should be used to achieve the desired result. Useful agents can be selected with the described assays.

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25

EXAMPLE 9

3-Isobutyl-1-methylxanthine (IBMX) is commonly used during cAMP assays (at a concentration of 500  $\mu$ M) to inhibit cAMP breakdown by the enzyme cAMP-phosphodiesterase. Thereby, one increases the levels of cAMP accumulating during the assay. A direct measurement of  $\mu$  receptor phosphorylation, using the epitope tagged  $\mu$  receptor gene and the  $^{32}$ P labeling assay, was

conducted by techniques analogous to those already described. IBMX at 500  $\mu$ M, supposed  $\mu$  receptor phosphorylation. IBMX at that concentration appeared to be more effective than H7 at 100  $\mu$ M because it  
5 prevented the naloxone cAMP overshoot, even when added only to the acute cAMP accumulation assay, and not as a pretreatment agent.

Thus, IBMX is active in preventing receptor phosphorylation and appears to be an inhibitor of the  $\mu$   
10 receptor kinase(s). This is of general importance, as this activity may also apply to receptors other than the  $\mu$  opioid receptor (e.g., the dopamine or muscarinic receptors). Moreover, IBMX is an alkylated xanthine, and thus, a prototype of a family of important drugs and  
15 chemicals, including theophylline, caffeine, theobromine. Among these one may find congeners with enhanced selectivity for the GPCR kinases over phosphodiesterases. Such an agent could be effective in the treatment of drug addiction, and other diseases that  
20 may be associated with elevated basal levels of receptor activity because of receptor phosphorylation.

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It is to be understood that while the invention has been described above in conjunction with preferred specific embodiments, the description and  
25 examples are intended to illustrate and not limit the scope of the invention, which is defined by the scope of the appended claims.

It is Claimed:

1. A method for screening G protein coupled receptors for constitutive activity, comprising;  
providing a plurality of G protein coupled receptors, a G protein coupled receptor kinase inhibitor, a phosphatase inhibitor, and a negative antagonist;  
5 determining a control value, the control value determinable by measuring the activity of the G protein coupled receptors after agonist pretreatment and subsequent removal in the presence or absence of a  
10 negative antagonist or agonist of the G protein coupled receptor;  
determining a kinase inhibitor value by exposing the G protein coupled receptors to an agonist  
15 of the G protein coupled receptors and the G protein receptor kinase inhibitor, and measuring the activity of the G protein coupled receptors following the removal of the pretreatment agents in the presence or absence of a negative antagonist;  
20 determining a phosphatase inhibitor value, the phosphatase inhibitor value determinable by pre-exposing the G protein coupled receptors to an agonist of the G protein coupled receptor and to the phosphatase inhibitor, and measuring the activity of the G protein  
25 coupled receptors in the presence or absence of a negative antagonist;  
comparing the control value with the kinase inhibitor value and the phosphatase inhibitor value;  
and,  
30 determining whether the G protein coupled receptors have constitutive activity as a result of phosphorylation by agonist pretreatment.



2. The method as in claim 1 wherein determining the activity of the G protein coupled receptor includes measuring cAMP levels.

3. The method as in claim 1 wherein determining the activity of G protein coupled receptors is by measuring an activity of G protein coupled receptor (GPCR) kinase as stimulated by a GPCR.

4. The method as in claim 1 wherein determining the activity of G protein coupled receptors includes measuring PI turnover.

5. The method as in claim 1 wherein determining the activity of G protein coupled receptors includes measuring GTPase and GTP exchange activity of the G protein.

6. The method as in claim 1 wherein determining the activity of G protein coupled receptors is by measuring the direct phosphorylation of the G protein coupled receptors.

7. The method as in claim 1 wherein determining the activity of G protein coupled receptors is by labelling the constitutively active G protein coupled receptors (GPCR\*) after agonist pretreatment.

8. The method as in claim 1 wherein the G protein coupled receptor kinase inhibitors are selected for the purpose of preventing GPCR\* formation.

9. The method as in claim 7 wherein the labelling is performed with radioactive atoms.

10. A method for assaying for the effects on G protein coupled receptor activity, comprising:  
providing a biological system including a plurality of G protein coupled receptors;  
5 exposing the G protein coupled receptors to a test composition; and,  
determining whether the G protein coupled receptors have constitutive activity by at least one effect of the test composition on the G protein coupled  
10 receptors.

11. The method as in claim 10 wherein determining the activity of the constitutively active G protein coupled receptors (GPCR\*) includes measuring cAMP levels, PI turnover, GTPase activity or GTP  
5 exchange.

12. The method as in claim 10 further comprising:  
classifying the activity of the test composition as a agonist, negative antagonist, or  
5 neutral antagonist, with respect to signalling and receptor phosphorylation.

13. The method as in claim 11 wherein the measuring step includes:  
determining a first cAMP value, the first cAMP value determinable by measuring the effects of a first  
5 portion of the receptors on cAMP production in the absence of agonist induced G protein coupled receptor activity;  
determining a second cAMP value, the second cAMP value determinable by measuring the effects of a  
10 second portion of the receptors on the cAMP production

while in a constitutively active state but being substantially free of agonist molecules;

determining a third cAMP value, the third cAMP value determinable by measuring the effects of the third portion of the receptors on cAMP production while (a) in  
15 a constitutively active state, (b) being substantially free of any agonist molecules, and (c) in the presence of a sufficient quantity of a negative antagonist or test compound to associate negative antagonist or test  
20 compound with substantially all the receptors; and,

combining a test portion of the receptors with a test composition and measuring the effects thereof on cAMP production as a test cAMP value.

14. The method as in claim 10 wherein determining the activity of the constitutively active G protein coupled receptors (GPCR\*) is by measuring an activity of a G protein receptor (GRK) kinase.

15. The method as in claim 10 further comprising:

determining inhibitors for G protein receptor (GRK) kinase.

16. The method as in claim 10 wherein determining the activity of the constitutively active G protein coupled receptors (GPCR\*) is by measuring the direct phosphorylation of the G protein coupled  
5 receptors.

17. The method as in claim 10 wherein the G protein coupled receptor is the D2 dopamine receptor.

18. The method as in claim 10 wherein the G protein coupled receptor is the m1 muscarinic receptor.

19. The method as in claim 17 wherein  
determining the activity of the constitutively active m1  
5 muscarinic receptor includes measuring phosphatidyl  
inositol levels.

20. A method of screening for agonists that  
prevent the formation of constitutively active G protein  
coupled receptors (GPCR\*), comprising:

5 providing a plurality of a G protein coupled  
receptors;

exposing a series of agonists of the G protein  
coupled receptor to the plurality of G protein coupled  
receptors;

10 determining the activity of the constitutively  
active G protein coupled receptors (GPCR\*) by measuring  
at least one effect of a given agonist on the G protein  
coupled receptors (GPCR\*); and,

15 selecting an agonist that prevents the  
formation of the constitutively active G protein coupled  
receptors (GPCR\*).

21. The method as in claim 20 wherein the G  
protein coupled receptor is the D2 dopamine receptor.

22. The method as in claim 10 wherein the G  
protein coupled receptor is the m1 muscarinic receptor.

23. The method as in claim 22 wherein  
determining the activity of the constitutively active m1  
muscarinic receptor includes measuring phosphatidyl  
inositol levels.

24. The method as in claim 22 wherein  
determining the activity of the constitutively active G

protein coupled receptors (GPCR\*) includes measuring cAMP levels.

25. The method as in claim 20 wherein determining the activity of the constitutively active G protein coupled receptors (GPCR\*) is by measuring an activity of a G protein coupled receptor (GPCR) kinase.

26. The method as in claim 20 wherein determining the activity of the constitutively active G protein coupled receptors (GPCR\*) is by measuring the direct phosphorylation of the G protein coupled  
5 receptors.

27. The method as in claim 20 wherein determining the activity of constitutively active G protein coupled receptors is by labelling the constitutively active receptors.

28. A method of treating a patient suspected of having taken an overdose of a narcotic analgesic, comprising:

5 selecting an agent determined to be a null antagonist for the suspected narcotic analgesic; and, administering the selected null antagonist for the suspected narcotic analgesic in a pharmaceutically effective amount.

29. The method as in claim 28 wherein the dose administered is effective to block narcotic agonist effects in addicted patients without inducing severe withdrawal in treating a narcotic overdose or to  
5 initiate withdrawal treatment.

30. The method as in claim 28 wherein the null antagonist administered includes D-Phe-Cys-Tyr-D-Trp-Orn-Tyr-Pen-Thr-NH<sub>2</sub>.

31. The method as in claim 28 wherein the null antagonist administered is nalorphine.

32. The method as in claim 28 wherein the null antagonist administered is CTAP (D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH<sub>2</sub>).

33. The method as in claim 28 wherein the null antagonist administered is D-Tic-CTAP (D-Tic-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH<sub>2</sub>).

34. A therapeutic method for treating a patient addicted to a narcotic drug, comprising:

5       selecting an agent determined to prevent and/or reverse constitutive activation of opioid  $\mu$  receptors; and

          administering a therapeutically effective amount of the selected agent to the addicted patient.

35. The therapeutic method as in claim 34 wherein the agent selected is 1-(5-isoquinoline-sulfonyl)-2-methylpiperazine dihydrochloride.

36. The therapeutic method as in claim 34 wherein the agent selected is 3-isobutyl-1-methylxanthine.

37. A therapeutic method for treating a patient addicted to a narcotic drug who is suffering from an overdose of the narcotic drug, comprising:

45

selecting an agent that does not block  
5 constitutive activity of opioid  $\mu$  receptors; and  
administering a therapeutically effective  
amount of the selected agent to the addicted patient.

38. The therapeutic method as in claim 37  
wherein the agent selected also is capable of reversing  
the constitutively activated state of opioid  $\mu$   
receptors to a normal  $\mu$  receptor state.

39. A therapeutic method for providing  
analgesia to a patient, comprising:  
selecting a kinase inhibitor; and,  
administering a narcotic analgesic in  
5 conjunction with the selected kinase inhibitor.

40. The therapeutic method as in claim 39  
wherein the kinase inhibitor selected is effective to  
retard development of tolerance to the narcotic  
analgesic administered.

41. The therapeutic method as in claim 40  
wherein the kinase inhibitor is selected for preventing  
the formation of constitutively active  $\mu$  opioid  
receptors.

42. A therapeutic for providing analgesia to  
a patient, comprising:  
selecting a  $\mu$  receptor agonist having a  
property of converting  $\mu$  receptors to their  
5 constitutively active state that is lower than morphine  
converts the  $\mu$  receptors to their constitutively active  
state; and,  
administering a therapeutically effective  
amount of the selected agent to the patient.

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US96/07375**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(6) :G01N 33/53; A61K 38/00

US CL :435/7.1, 7.21; 514/2

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/7.1, 7.21; 436/501; 514/2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WANG et al. Agonist Induced Constitutive Activation of the $\mu$ Opioid Receptor By Phosphorylation. Regulatory Peptides. 1994, Vol. 54, No. 1, pages 323-324, see entire article.	1-27
X, P	BILSKY et al. Effects of Naloxone and D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH <sub>2</sub> and the Protein Kinase Inhibitors H7 and H8 on Acute Morphine Dependence and Antinociceptive Tolerance in Mice. The Journal of Pharmacology and Experimental Therapeutics. April 1996, Vol. 277, No. 1, pages 484-490, see abstract.	1-42

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A" document defining the general state of the art which is not considered to be of particular relevance	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E" earlier document published on or after the international filing date	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Z" document member of the same patent family
"O" document referring to an oral disclosure, use, exhibition or other means	
"P" document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

26 AUGUST 1996

Date of mailing of the international search report

30 SEP 1996

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# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US96/07375

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WANG et al. Constitutive $\mu$ Opioid Receptor Activation as a Regulatory Mechanism Underlying Narcotic Tolerance and Dependence. Life Sciences. 1994, Vol. 54, No. 20, pages 339-350, see entire article.	1-42

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## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US96/07375

### B. FIELDS SEARCHED

Electronic data bases consulted (Name of data base and where practicable terms used):

STN, APS

search terms: G protein coupled receptors, opioid receptors, D2 dopamine receptor, m1 muscarinic receptor, constitutive, kinase inhibitor, phosphatase inhibitor